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부 용출 · 정 진

The Light Quality Dependence of Photoresponse of Mud Fish (*Misgurunus mizolepis* Günther)and the Chromophores Photogenerating Active Oxygen in its Skin Tissues

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Abstract

A photoresponse that results in organisms dispersing from a region of bright light, generally termed photodispersal, is frequently observed in some fishes notably including mud fish (Misgurunus mizolepis GÜNTHER). The primary assumption for this study was that the photodispersion may result from the behavioral strategies of fishes aimed to avoid illumination conditions that could injure the cells in skin tissues via photodynamic sensitization reactions. Here we present some preliminary results that seem to support this assumption : (1) the locomotive action of dark-adapted mud fish was triggered by the onset of illumination with light : (2) blue light (400-500nm) was much more effective in bringing about the locomotive activity than yellow (550-650nm) and red (650-800nm) lights : (3) two blue light absorbing pigments, which photogenerate activated oxygen species, were separated from the skin tissues of mud fish, one of these being identified as riboflavin.

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INTRODUCTION

When organisms are subjected to light stimulus, they respond and adapt. A response that results in organisms dispersing from a region of bright light is referred to as photodispersal, the behavioral consequence of negative phototaxis and positive photokinesis(1). Photodispersing is frequently observed in some fishes; mud fish(Misgurunus mizolepis GÜNTHER) is one of these. Nontheless, the photobiological nature of this photoresponse of fishes is remained poorly understood and information from which the underlying mechanisms might be deduced is extremely scanty.

As is well known, the primary event of any photobiological phenomenon is photoreception, a photophysical process of light absorption by certain intracellular chromophores. Retinal photoreception is the most common and relatively well understood in the animal kingdom. However, some species are known to have extraretinal photoreception. In fishes, for instance, pineal organ which is located on the dorsal part of the brain has been proposed as a predominant extraretinal photoreceptor(2,3), while extraretinal nonpineal photoreception has been also suggested in several fishes(4, 5).

Assuming that a photoreception which can lead to photosensitization reactions in cells might occur in the skin tissues of mud fish and that photodispersal results from a behavioral strategy evolved in the fish to avoid the hazardous light-induced reactions of cellular components, we attempted to investigate this subject. In the present study the results demonstrate that mud fish shows photoresponse to a greater extent in blue light(400-500nm) than yellow(550-650nm) and red(650-800nm) lights, that there is a nonretinal photoreception which seems more effective with blue light in bringing about photoresponse, and that there exist at least two blue light-absorbing pigments photogenerating active oxgen in the skin tissues, one of which appeared to be riboflavin.

MATERIALS AND METHODS

Measurement of light-induced locomotion activity of mud fish. The locomotion activity of mud fish was monitored by the electropotential method, as described by Spoor(6), that measures the potential difference between the two stainless steel electrodes set at opposite ends of the experimental chamber(20x20x20cm) made of flexiglass.

The potential change elicited by the movement of the fish was recorded on a Shimadzu strip chart recorder. Before the measurements, the fish(10 animals for each experiment) was acclimatized for at least 12 hours in the chamber supplied with tap water of 20±2℃ at a flow rate of 0.35 l min-1 in the dark without feeding. Light stimulus was delivered to the dark-adapted fish from the front side of the chamber at the same time of the day to minimize circadian variations in response. In order to evenly illuminate the whole body of fishes a plate mirror was placed on the backside of the chamber.

The colored lights were obtained from a 1 KW Xe-arc lamp(Osram) by use of various

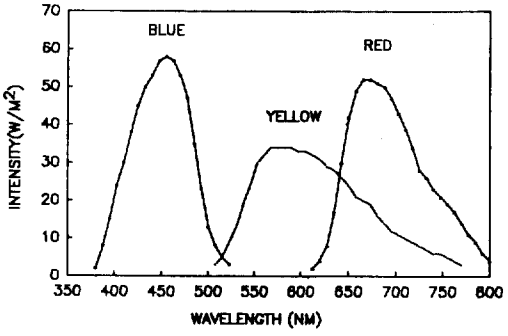

Fig. 1. Spectral distribution of energy fluence rate of blue, yellow and red light used in the experiment.

optical filters: the spectral distrbution of light fluence rate of the colored(blue, yellow and red) lights were as shown in Fig.1. The light intensities were controlled with neutral density filter and an adjustable power supply. The light fluence rate near the center of the chamber was measured by using a remote probe of a Macam quantum radiometer/photometer. For the experiments with blind mud fish ophthalmectomy was performed by dissecting the opaque tissue of eyes with a syringe needle and removing the eye balls. All operated fishes were kept for 5-7 days in a laboratory tank before the commencement of measurements. Mud fishes were obtained from a local supplier in Suwon, Korea: body weight was in 12-20g and length was in 10-15cm. They were kept in the laboratory tank with feeding.

Extraction and purification of photosensitizing pigments from the skin tissues of mud fishes. The fish was decaptitated and the skin tissues were immediately taken from the truncks. Since it was found in preliminary experiments, in which extraction with an aquous medium, methanol and n-hexane were performed respectively, that methanolic extraction is the most efficient in separating photosensitizing chromophores from the skin tissues, we used methanol as the extraction medium. The collected skin tissues(100g fresh weight) were blended in 1 liter of methanol for 30sec and then filtered through two layers of cheese cloths. The filtrate was centrifuged(10,000 x g for 5min) and the supernatant was evaporated at 40℃ under reduced pressure to dryness. The dried matter was dissolved in a chromatographic solvent(5ml) of n-butanol/ethanol/water (85/10/5) and applied to a silica gel column(Ø2x20cm, Kieselgel 60G, Art. 9385, Merck). Two active fractions with elution volume of 95-117ml and 131-162ml, which photogenerate active oxygen(superoxide radicals and singlet oxygen) were obtained. Further purification of the active ingredients was performed on a Sephadex LH-20 column(Ø1 x 43cm, Pharmacia) by using chloroform/methanol (1/1).

Assay for the photogeneration of superoxide and singlet oxygen The production of superoxide by photosensitizing pigments was monitored by the reduction of nitro blue tetrazolium(NBT), as described Beauchamp(7), while the photosensitized generation of singlet oxygen was measured by the imidazole plus N,N-dimethyl-4-nitrosoaniline(RNO) method developed by Kraljic and Mohsni(8). For this, 0.5ml of each eluted fraction was admixed with an equal volume of 0.5M K-PO4 buffer(pH 7.8) containing either 300μ M NBT or 10μ M RNO plus 16mM imidazole and then irradiated with white light(visible light only) from a 1KW Xe-Arc lamp(UV and IR components of the whole light were effectively filtered-off by use of a UV-IR cut-off filter).

The formation of formazan resulting from the NBT reduction by superoxide and the bleaching of RNO resulting from the singlet oxygen-imidazole-RNO reaction were followed spectrophotometrically 530nm and 440nm respectively. Experiments were conducted at 20℃ under safety room light.

Chromatograhpic and spectroscopic analysis of the isolated pigments Two active fractions from a Sepadex LH-20 column were subjected to the measurements of their UV-Visible absorption and fluorescence spectra. In addition, some chromatographic parameters were also determined by reversed phase HPLC as well as by TLC. For HPLC, the eluent(methanol/0.1M ammonium acetate, 4/6) was flowed through a Lichrosorb RP-18 column(Φ4.6x250 mm) at a rate of 1ml/min and the effluent was monitored by either absorbance at 420 nm or fluorescence intensity(λ ex=420 nm and λ em>490). For TLC silica gel G-60 and a mixture of n-butanol/ethanol/water(85/10/5) were used as an adsorbent and a developing solvent, respectively.

Instruments and chemicals. Throughout the work absorbance and absorption spectrum were measured with a Diode Array Spectrophotometer HP 8452A(Hewlette Packard) and fluoresence specta with a spectrofluorometer SPF 5000C(SLM Ins. INC). A HPLC system with a SP 8000 hplc pump and a SP 4270 integrater (Spectraphysics), also equipped with a spectroflow 757 absorbance detector(ABI Analytical Kratos Division) and a fluorescence detector model 420-420AC (Waters) was employed for high pressure liquid chromatography. Chemicals were purchased from either Sigma, Merck, or Fluka chemie AG; these were used without further purification.

RESULTS AND DISCUSSION

Photoreponse of mud fishes: Light-induced locomotion activity has been used as an indicator of the photoreception of fishes, as it is easy to measure and does not require any restraint forced upon the animals(9). Being kept in the dark for a sufficient time, the fish did not show any locomotive activity detectable at least by the potential change occuring in the chamber. Light stimulus delivered to the quiescent fish caused an immediate moving activity. However, this stimulus appeared to be dependent not only on energy fluence rate but also on light quality. Blue light was much more effective in producing photoresponse in the fish than red light, while the extent of the response was increased with the increased intensities of light.

As can be seen in Fig. 2, the energy fluence rate of 15w/m^2 was strong enuogh for blue light to elicit the locomotion activity in mud fishes, but it is apparently too weak for red light to stimulate the fish. Red light at 200w/m^2 exerted a stimulating

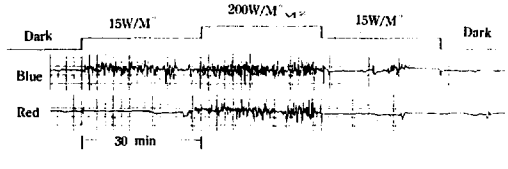

Fig. 2. Dependence of light-induced locomotion activity of mud fish on energy fluence rate of blue and red lights. The light intensity was changed every 30 min as indicated.

effect on the fish causing photoresponse whose extent seemed more or less same with that of the response to blue light at only 15w/m^2.

The light quality dependence of photoresponse was further demonstrated by the results shown in Fig.3; one can see that photosensitivity of intact mud fish decreased in the order of blue, yellow, and red lights. Fig.3 also shows that blind mud fish responded to light stimulus, although the response was much weaker than that of intact one, indicating that indeed there exists a extraretinal photoreception in mud fish.

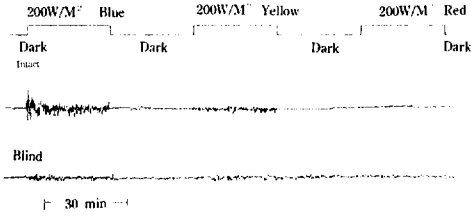

Fig.3. Dependence of light-induced locomotion activity of mud fish on light quality, as measured with intact and blind mud fishes.

Further, this result seemed to implicate that blue light is again more effective as a stimulus for blind fish than yellow and red lights, as observed in intact one. Imaginable as it may be, however, this result in itself did not provide evidence that the light absorbing pigments involved in the extraretinal photoreception would be chemically identical with that of vision photoreception. We rather imagine that this extraretinal photoreception would lead to generate a certain signal transmitted to brain, alarming the forthcoming dangerous situations and that the sensory transduction could be aggravated when it was linked with vision photoreception in intact fish.

Then a question arises: what kind of dangerous situations is it? We assume that it is the overproduction of activated oxygen species produced through photosensitization reactions in skin cells; for some of activated oxygen species are so reactive that they can degrade a variety of crucial components of cells(10,11).

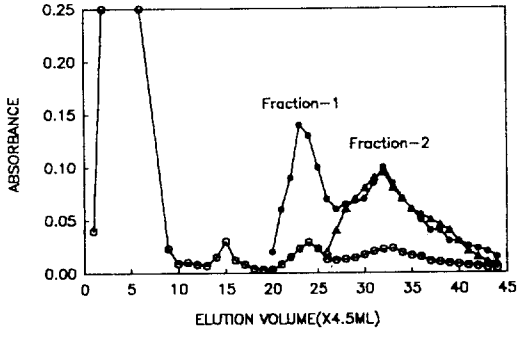

Fig.4. Elution profile of the methanolic extract from silica gel column. The chromatograms were prepared based on the measurements of absorbance at 420nm(○) NBT reduction(●)and RNO bleaching(▲).

Photosensitizing pigments in the skin tissues of mud fish

Column(silica gel) chromatographic measurements revealed that at least two pigments acting as photosensitizers are present in the methanolic extract of mud fish skin tissues. One of these, when exposed to visible light under aerobic conditions, produced both singlet oxygen and superoxide radicals, whereas the other photogenerated only superoxide, as shown in Fig. 4. The pigments, further purified by

Sephadex LH-20 column chromatography, absorb blue light with absorption maxima at 450nm and 420nm respectively. The purified pigment initially contained in the fraction-2 (in Fig. 4) showed absorption spectrum and fluoresence emission and excitation spectra that are almost identical with those of riboflavin(see Fig. 5).

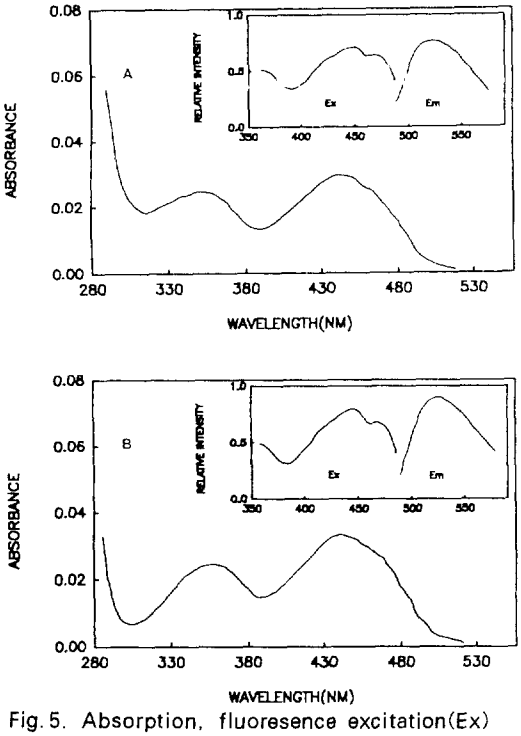

Fig. 5. Absorption, fluoresence excitation(Ex) and emission(Em) spectra of a pigment isolated from mud fish skin tissues photogenerating active oxygen in (A) and those of authentic riboflavin in (B).

This observation prompted us to speculate that the pigment is riboflavin. Since all flavins including FMN and FAD show practically identical absorption and fluoresence spectra(although fluoresence quantum yield of FAD is very low), however, the pigment could be another compound having a flavin chromophore rather than free riboflavin. Thus we conducted both silica gel TLC with riboflavin, FMN and FAD, and HPLC with authentic riboflavin in order to prove whether the pigment is riboflavin.

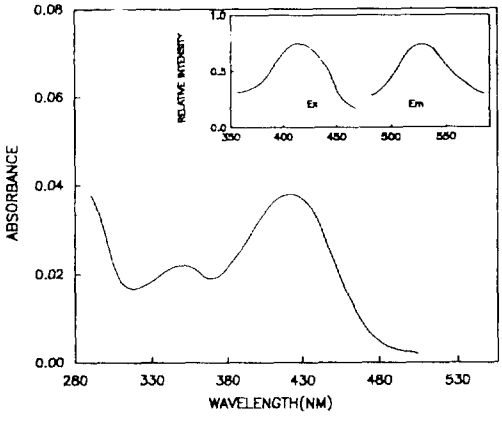

Fig. 6. Absorption, fluoresence excitation(Ex) and emission(Em) spectra of another pigment from mud fish skin tissues photogenerating superoxide radicals.

The results from the TLC experiment(data not shown) as well as from the HPLC measurement(Fig. 7) clearly indicated that it is most likely riboflavin. Furthermore, the

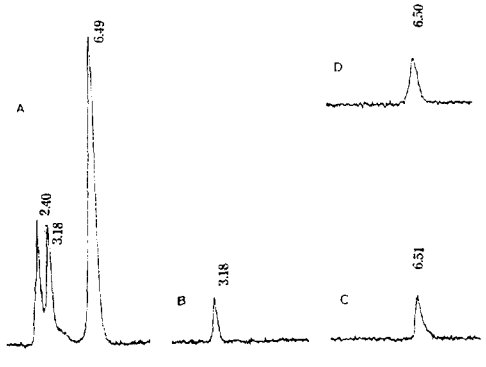

Fig.7. Typical high pressure liquid chromatograms of the methanolic extract of the mud fish skin (A), fraction-1 (B) and fraction-2. The chromatogram of authentic riboflavin is shown in (D). Signals were obtained by using fluoresence detector(λ ex+420nm, λ em>490nm). As to fraction-1 and 2, see the Fig.4.

fact that riboflavin can photogenerate both singlet oxygen and superoxide(12) is also consistent with the suggestion that the pigment in the fraction-2, which produced both of the activated oxygen species upon irradiation, is riboflavin.The content of free riboflavin in skin of mud fish was estimated to be about 40μ g/100g fresh tissues.

For the other pigment contained in the fraction-1, which photogenerated superoxide radicals, no information is available at present except for the absorption and emission spectra(Fig.6) measured in this work. Extensive structural analysis performed by employing such elaborate techniques as high resolution IR, NMR, and Mass spectrometry could disclose its

CONCLUSIONS

To our knowledge this type of study, attempting to relate photodispersing response of a higher animal to the photodynamic sensitization occuring in skin tissues, is the first ever carried out. Although the results presented here are not sufficient enough to produce any conclusive suggestion, they still seem to implicate that photogeneration of active oxygen by endogenous sensitizing pigments is involved in photoresponse of mud fish. The involvement of active oxygen generation in photoresponse of some organisms is not unusual. For instance, according to the work by Ueda et al(13), in plasmodia of an albino strain of *physarum polycephalum* active oxygen, particulary superoxide radicals appear to trigger UV and blue light photoavoidance. Existence of free riboflavin, a well known photosensitizer, in skin tissues is never frequently observed in the higher animal kindom. Thus, the isolation of free flavin of significant amount from the skin of the light-avoiding fish seems to circumstantially support the aforementioned implication.

요 약

이동성의 생명체는 자신에게 불리한 환경을 피해 이동할 수 있는 자기 방어 기구를 갖고 있다. 본 연구에서는 미꾸라지(Misgurunus mizolepis G ÜTHER)가 보이는 photodispersal 현상도 광증감반응을 통해 피부세포를 손상시킬 수 있는 해로운 광자극을 피하기 위한 일종의 방어 전략일 것이라 가정하고 이를 뒷받침할 수 있는 다음과 같은 결과들을 얻었다.

첫째, 암소에서 적응된 미꾸라지는 빛에 노출되면 즉시 운동성의 증가를 보이고 황색 또는 적색광에 비해 특히 청색광에 민감하게 반응하였다.

둘째 눈을 멀게한 미꾸라지도 광반응을 보였으며 청색광에 비교적 예민하게 반응하였다.

셋째, 미꾸라지 피부조직에는 활성산소를 생성시킬수 있는 청색광 흡수 광증감색소가 최소 두종류가 존재하고 있었으며 그 중 하나는 리보플라빈으로 동정되었다.

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